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Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*

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Abstract

When ectotherms are exposed to low temperatures, they enter a cold-induced coma (chill coma) that prevents resource acquisition, mating, oviposition, and escape from predation. There is substantial variation in time taken to recover from chill coma both within and among species, and this variation is correlated with habitat temperatures such that insects from cold environments recover more quickly. This suggests an adaptive response, but the mechanisms underlying variation in recovery times are unknown, making it difficult to decisively test adaptive hypotheses. We use replicated lines of *Drosophila melanogaster* selected in the laboratory for fast (hardy) or slow (susceptible) chill-coma recovery times to investigate modifications to metabolic profiles associated with cold adaptation. We measured metabolite concentrations of flies before, during, and after cold exposure using NMR spectroscopy to test the hypotheses that hardy flies maintain metabolic homeostasis better during cold exposure and recovery, and that their metabolic networks are more robust to cold-induced perturbations. The metabolites of cold-hardy flies were less cold responsive and their metabolic networks during cold exposure were more robust, supporting our hypotheses. Metabolites involved in membrane lipid synthesis, tryptophan metabolism, oxidative stress, energy balance, and proline metabolism were altered by selection on cold tolerance. We discuss the potential significance of these alterations.

Keywords

energy balance; thermal limits; insect; winter; correlation networks; NMR-based metabolomics

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Introduction

Bioenergetics, or energy flow within organisms, interacts with temperature to constrain species distributions and influence global patterns in species abundance (Clarke and Gaston 2006). Rates of energy flow through metabolic pathways are strongly dependent on temperature because decreases in temperatures slow rates of biochemical reactions within the cells of an organism (Logan 1982). Normal physiological function requires organisms to precisely and dynamically balance energy supply and demand, but metabolic pathways differ in susceptibility to thermodynamic effects (Clarke and Fraser 2004). This creates a considerable challenge for ectotherms, whose body temperatures fluctuate with environmental temperatures, to maintain energetic and metabolic homeostasis under fluctuating thermal conditions. Species distributions can thus be driven not only directly by exposure to lethal temperatures, but also indirectly through repeated or long-term exposures to sub-lethal temperatures that lead to energetic failures (Fly and Hilbish 2013).

Energetic failures result from an inability to supply or use sufficient energy to perform physiological functions necessary to support life. In marine organisms, energetic failures at temperatures outside the thermal window for growth and reproduction are driven by inability to supply oxygen and thus maintain aerobic scope for normal physiological function (oxygen- and capacity-limitation of thermal tolerance, Portner 2001). Oxygen-delivery limitations result in an increasing reliance on anaerobic metabolism and the development of low cellular energy levels (Gibbs free energy of adenosine triphosphate [ATP] hydrolysis, Zielinski and Pörtner 1996). However, terrestrial insects have air-filled tracheal tubes that supply oxygen directly to tissues without reliance on a circulatory system, and there is currently little support for oxygen and capacity limitation of thermal tolerance in these taxa (Klok et al. 2004; Stevens et al. 2010; Macmillan et al. 2012a; McCue and De Los Santos 2013). The “weak link” in the energy supply and demand chain that explains variation in performance at extreme temperatures thus remains to be identified in terrestrial insects (and other terrestrial ectotherms).

In insects, low temperatures induce a reversible loss of neuromuscular coordination (chill-coma) during which mating and foraging is precluded and predators cannot be avoided. Recovery time from chill-coma is a commonly used metric of cold hardiness. Because chill-coma recovery time is usually assessed after exposure to mild low temperatures that do not cause cold shock (i.e. direct chilling injury that develops rapidly as a result of protein denaturation, membrane phase transitions, or cell death; Koštál et al. 2011a), variation in recovery time likely results from variation in either the ability to maintain homeostatic processes in the cold or the rate of recovery of those processes upon rewarming. Ionic regulation is a critical homeostatic processes compromised in the cold.

While an insect is in chill coma, membrane equilibrium potentials are progressively depolarized as extracellular [K^+] increases due to a loss of ionic regulation (Macmillan and Sinclair 2011a; Andersen et al. 2013; Findsen et al. 2013). In the fall field cricket, this progressive loss of ionic homeostasis is accompanied by the development of chilling injury, and, upon rewarming, osmotic and ionic equilibrium are gradually restored (MacMillan and Sinclair 2011b). Recovery (the resumption of coordinated movement) coincides with the

restoration of osmotic and ion concentration gradients, and the speed of recovery of ion homeostasis correlates positively with cold hardiness (Findsen et al. 2013). Complete recovery of ionic and osmotic homeostasis following cold exposure is an energetically expensive process that can take several hours (MacMillan et al. 2012b).

Although it is clear that the maintenance and recovery of ionic homeostasis plays a proximate role in modulating cold hardiness, the mechanisms underlying evolutionary variation in ion homeostasis are still unknown. Energy supply limitations may compromise the function of ion-motive pumps that regulate ionic and osmotic homeostasis, but to date there are few indications that ATP supply is compromised in insects at low temperatures. Aerobic metabolism and ATP supply is maintained down to the freezing point of the body tissues (~ −10 °C) in freeze-tolerant pupae of the gall-fly, *Eurosta solidaginus* (Storey and Storey 1981), and the firebug, *Pyrrhocoris apterus*, maintains its energy state right until death from cold injury (Koštál et al. 2004). Some studies document an ATP decline in insects at low temperatures (Pullin et al. 1990; Dollo et al. 2010), but this decline develops slowly and may represent secondary effects rather than the cause of energetic failure. In other cases, ATP accumulates during prolonged or lethal cold exposures (Coulson et al. 1992; Macmillan et al. 2012a), suggesting that the ability to utilize, rather than synthesize, ATP may be reduced in the cold.

Low temperatures may inhibit energy-demanding processes directly through thermal effects on ion-motive pumps or metabolic enzymes, or indirectly via modulation of the lipid membrane environment of cells and organelles (Koštál et al. 2011a; Macmillan and Sinclair 2011a; Findsen et al. 2013). Alterations to lipid membrane fluidity will affect rates of proton leak and thus oxidative stress, and performance of membrane-bound proteins such as ion-motive pumps and metabolic enzymes. Whether the effects of temperature are direct or indirect, alterations to energy homeostasis will occur and these alterations should be associated with distinct metabolomic fingerprints.

Metabolomics provides a snapshot of a portion of the metabolome at a point in time, and thus does not provide information on flux through underlying pathways. However, correlations among metabolites across biological samples at steady state can be used to infer the relative importance of various enzymatic reactions, and the underlying regulatory structure of the system as a whole (Weckwerth and Morgenthal 2005). The metabolic network of an organism is the complete set of biochemical reactions occurring within its cells, including all metabolic pathways and regulatory interactions among them. Interdependencies arise between metabolites that interact through the metabolic network as a result of fluctuations induced by environmental (e.g. temperature) or intrinsic variability (e.g. enzyme concentration) (Steuer 2006). Reaction interdependencies generate patterns of correlations among metabolites that provide a fingerprint of the underlying physiological state of the organism (Camacho et al. 2005; Morgenthal et al. 2006). Therefore, if cold exposure disturbs the physiological state of an organism, we expect alterations to the structure of correlation-based metabolic networks. The degree of disruption to these correlation networks may be associated with underlying genetic variation in chill-coma recovery time.

Although no studies have investigated the maintenance of metabolic networks during cold exposure, there is evidence for loss of metabolic homeostasis in insects at low temperatures (Overgaard et al. 2007; Colinet et al. 2012). This manifests in increases in free amino acid concentrations that may result from cold-induced protein breakdown (Lalouette et al. 2007; Koštál et al. 2011b; Colinet et al. 2012) or an imbalance between glycolysis and the tricarboxylic acid (TCA) cycle (Michaud and Denlinger 2007; Koštál et al. 2011b). Cold exposure also increases sugars (Overgaard et al. 2007; Colinet et al. 2012; Teets et al. 2012) and sugar alcohols such as glycerol and sorbitol (Michaud and Denlinger 2007).

It is clear that both acclimation and adaptation can modulate the degree of cold-induced disruption to homeostatic processes. Acclimation to low temperatures improves both ionic (Findsen et al. 2013) and metabolic homeostasis in the cold (Overgaard et al. 2007; Colinet et al. 2012). At the whole organism level, chill-coma recovery time is evolutionarily labile - latitudinal variation within and among species are consistent with local adaptation (David et al. 2003; Sinclair et al. 2012), and cold hardiness traits respond to artificial selection in the laboratory, including chill-coma recovery time (Mori and Kimura 2008; Goto et al. 2010). However, we still do not know whether genetically based variation in the ability to maintain metabolic processes in the cold underpins variation in susceptibility to cold stress. A strong evolutionary perspective on this question has potential to advance our understanding of the mechanisms of low temperature tolerance in insects.

Here, we test the hypothesis that fly lines selected for cold-hardiness will maintain metabolic homeostasis to a higher degree than cold-susceptible flies during cold exposure and recovery. Specifically, we predict that the metabolic networks of cold-hardy flies will be more resistant to cold stress, maintaining their structure better during cold exposure than the metabolic networks of cold-susceptible flies, and that the network structure of cold-hardy flies will also be more tolerant of cold stress, re-establishing their structure more quickly during recovery from cold stress. We use replicate experimental evolution lines of *Drosophila melanogaster* selected in the laboratory for fast or slow recovery from chill-coma. These lines originate from a natural population in Raleigh, North Carolina, and have stable and genetically based divergence in chill-coma recovery. We use nuclear magnetic resonance (NMR) spectroscopy to profile the water-soluble metabolome, demonstrating that selection for rapid chill-coma recovery results in improved metabolic homeostasis and more robust metabolic networks.

Materials and Methods

FLY STOCKS AND EXPERIMENTAL DESIGN

Flies from two base populations, each collected at the same locale in Raleigh North Carolina, were selected for 31 generations for fast (hardy) or slow (susceptible) recovery from chill coma at Kansas State University, and thereafter maintained at 25 °C (Supporting Information). These two base populations gave rise to two independent lines each of hardy and susceptible flies that were used in all metabolomics experiments. Flies for this study were reared at University of Florida at 25 °C on a 12:12LD cycle in 235 mL bottles on molasses-cornmeal-yeast medium under controlled density, achieved by allowing 10 females (accompanied by 5 males) to oviposit for 48 h. This resulted in uncrowded

conditions and relatively synchronous emergence. On day 12 following the beginning of oviposition, emerged flies were cleared and discarded. Twenty-four hours later emerged flies were transferred to holding bottles for 24 h to ensure mating, and groups of 20 females were sorted under light CO_2 anesthesia (<5 min) and left to recover for >48 h before use in experiments (yielding 5–8 day-old mated females).

Eight replicate pools of 10 flies from each line were frozen in liquid nitrogen at one of three time points: 1) before cold exposure, 2) at the end of a 3 h exposure at 0 °C, and 3) after 5 minutes recovery at room temperature following a 3 h exposure at 0 °C. To perform the cold exposure, flies were tapped without anesthetic into an empty vial and immediately placed in an ice-water slurry. Chill-coma recovery times were measured on 20 individuals from each line on the day metabolomics samples were collected (Supporting Information).

NMR SPECTROSCOPY

Samples were sent on dry ice to Claflin University for NMR analysis. Pools of 10 female flies (3–5 mg) were lyophilized from frozen (48 h), weighed for dry mass, and their polar metabolites were extracted (Supporting information). NMR spectra were recorded at 298 K on a Bruker Avance™ III spectrometer operating at 700 MHz equipped with a room temperature 5-mm triple resonance probe. Standard ^{1}H 1D (zg), first increment of presat-NOESY (noesypr1d), and two-dimensional ^{1}H-^{13}C edited heteronuclear single quantum correlation (HSQC) experiments were recorded with 2.91s acquisition times and 4s recycle delay (Supporting Information).

We tentatively assigned metabolites using a combination of the Chenomx NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada), chemical shifts of metabolites in Biological Magnetic Resonance Data Bank (BMRB) (http://www.bmrb.wisc.edu/metabolomics/), and other published chemical shift data (Bundy et al. 2007; Duarte et al. 2009; Lee et al. 2009; Triba et al. 2010). Metabolite identifications were confirmed where possible using proton carbon couplings derived from HSQC experiments (Table S1), and compounds were quantified using the intensity of a single peak chosen for minimal overlap with other compounds (Supporting Information, Fig. S2, Table S1). We validated 6 key compounds (proline, β-alanine, tyrosine, histidine, phosphocholine, and 3-hydroxykynurenine) by spiking in 100 μL of standards (Sigma Aldrich, St Louis, MO) to a concentration of 10x the Chenomx-determined concentration for each metabolite. In all 6 cases there was good evidence that the peak used for quantitation belonged to the compound in question (Fig. S1). We normalized peak integrals to the integral of our internal standard (TMSP, Supporting information), and normalized other experimental factors (including dry mass of flies) using a mixed model framework (Jauhiainen et al. 2014), as described in the following section.

DATA ANALYSIS

Survival analysis and general linear models—Statistical analyses were conducted in R 2.15.3 (R Core Team 2013) unless stated otherwise. We assessed differences among the lines in recovery times using accelerated failure time models (“survreg”) with right-censored data in the package “survival” (Therneau 2013). Accelerated failure time models test the hypothesis that a covariate influences the rate of change in the proportion of individuals

surviving over time (in this case, “surviving” corresponds to “remaining in chill-coma”). Accelerated failure time models are preferable to commonly used proportional hazard models because they are fully parametric, robust to omitted covariates or choice of probability distribution, provide better predictive power, and are easier to interpret (Keiding et al. 1997; Lambert et al. 2004; Sayehmiri et al. 2008). We compared models with weibull, exponential, gaussian, logistic, log-normal, and log-logistic error distributions, and selected the model with the lowest Akaike Information Criterion (AIC). We subsequently simplified the model stepwise by pooling lines and retained the simpler, pooled model if $\Delta AIC < 2$, until no further simplification was possible.

To test the effect of cold exposure and selection regime on metabolite levels, we fitted general linear models to each metabolite with base population as a random effect; cold exposure, selection regime, and their interaction as fixed effects; and dry mass of the combined flies in each sample as a covariate. Thus, significant fixed effects account for the independent replicates of the selection process. We controlled the experiment-wide false discovery rate at $Q < 0.05$ (Benjamini and Hochberg 1995). We performed principal components analysis using the *prcomp* function (Team 2013) on z-scored data. The input data were residuals from a regression of mass on each metabolite concentration to remove effects of mass from this analysis. The scores on each principal component accounting for > 10% of variance were analyzed for effects of selection and time using general linear models as described above.

Supervised classification and separation—We next fit statistical models that discriminate hardy from susceptible flies using combinations of metabolites and evaluated the robustness of the resulting models. All separation and classification tests were performed using MATLAB vR2013a (Mathworks, Natick, MA, USA) using libSVM implementation (Chang and Lin 2011) of Support Vector Machines (SVM) (Vapnik 1995). SVM was used as the building block of a prediction algorithm that searches for the minimum number of metabolites that can give perfect classification and separation on the training data (Supporting Information). The approach is incremental and exhaustive, in that all k–tuples for $k=1,\ldots,n$ of the n metabolites are checked to determine whether they can separate the training set samples. For each k–tuple, 1000 repetitions of a 10-fold cross validation are executed. The 10-fold cross validation process consists of dividing the data set into 10 parts (folds). At each step, one fold is used for testing and the remaining 9 are used for training. The training is repeated 10 times, each time with a different fold. Each time the accuracy of classification, that is the percentage of test points correctly assigned to their class by the model, is computed and the classification accuracy of the cross validation is the average of the accuracies computed for each fold. This process is repeated 1000 times, and accuracies are averaged to give the final accuracy for each k–tuple. The searching process is stopped when at least one k–tuple has an overall classification accuracy of 100%, so that the maximum value of k is equal to that which returns at least one k-tuple with 100% accuracy (although there may be many more). Once these models had been built, we performed a blind validation study by removing the labels from 12 vials of flies, measuring the metabolites and using the models to assign group membership, then testing our assignments (Supporting Information).

Time series analysis—We used Short Time Series Expression Miner (STEM) (Ernst and Bar-Joseph 2006) separately for hardy and susceptible flies (with the second selection population designated a repeat of the experiment) to identify clusters of metabolites with similar temporal profiles. We grouped profiles whose pairwise correlations (r) exceeded 0.7. This generated a set of 5 distinct temporal profiles (across both selection regimes) to which metabolites passing filtering criteria (described below) were assigned. We $\log_2$-normalised values relative to the "before" time point, and filtered metabolites that did not show a minimum absolute expression change exceeding 0.2, or whose temporal profile was not correlated across biological replicates of each selection regime ($r < 0.6$). All parameters were optimized through an iterative process on pooled data from all lines to return a moderate number of profiles, and to neither retain nor filter all metabolites. We used the resulting parameters to separately build profiles for hardy and susceptible flies. We performed a permutation test to determine which profiles had significantly more metabolites assigned than would be expected by chance. We used a chi-squared contingency table test on numbers of metabolites that were altered by cold compared to the numbers of metabolites preserved/unchanged throughout cold exposure for hardy and susceptible flies (function "chisq.test" in R base package).

Correlation network analysis—We calculated first order partial correlations separately at each time point for hardy and susceptible flies using the "GeneNet" package (Opgen-Rhein and Strimmer 2007). Partial correlations reduce the influence of transitive correlations and estimate pairs of metabolites that are interacting directly, or through an unobserved metabolite(s) (Steuer 2006). In this analysis, replicate selection populations were pooled to provide sufficient power to detect correlations, thus significant partial correlations are those that are conserved across both replicates of the selection procedure. To construct partial correlation networks, we used a static shrinkage estimator and retained only significant partial correlations ($Q < 0.1$). We visualized partial correlation networks as nodes (metabolites) connected by edges (significant partial correlations) using a force-directed layout in Cytoscape v.2.8.3 (Smoot et al. 2011). We calculated network parameters in Cytoscape for hardy and susceptible networks at each time point.

We then constructed differential correlation networks, to determine which correlations were altered by selection on cold hardiness. We calculated correlation matrices of Pearson's correlation coefficients (r) for all pairwise combinations of metabolites separately for hardy and susceptible flies, pooling biological replicates and time points to give us sufficient power to detect correlations (n=48 each for hardy and susceptible). The effect of pooling across time points here will be to limit our detection of correlations to those that are robust across the entire cold exposure, on aggregate. We transformed the r-values to approximate a normal distribution using a z-transform, then compared the matrices of hardy and susceptible flies using a t-test to identify conserved and altered correlations (and FDR-corrected the resulting p-values) (Morgenthal et al. 2006). For any correlation that was significantly altered between hardy and susceptible flies ($Q < 0.1$), we calculated the change in the correlation coefficient (Δr) for correlations that retained the same sign, or the sum of the absolute values of the correlation coefficients ($\Sigma|r|$) for correlations whose direction was reversed. We summed the absolute values of Δr and $\Sigma|r|$ across rows of the covariance

matrix to give a metric of the magnitude of alteration to the correlation structure of each metabolite as a consequence of selection on cold hardiness. We performed hierarchical clustering on resulting differential correlation matrices using the function “heatmap.2” (package “gplots”,(Warnes 2012) to aid visualization.

Results

SUMMARY OF RESPONSE TO COLD EXPOSURE AND SELECTION

All flies were in chill-coma at the end of a 3h exposure to 0 °C and hardy flies recovered significantly faster (line 1 = 6.1, line 2 = 5.8 min) than susceptible flies (line 1 = 12.4 min, line 2 = 23.7 min; Fig. 1A). The best accelerated failure time model to fit the chill-coma recovery times after the 3h cold exposure had a log-logistic error distribution with hardy lines pooled and susceptible lines separate (χ^2=164.55, p<0.0001). By 5 minutes of recovery ~40% of tolerant flies had regained the ability to stand, whereas all susceptible flies were still in chill coma (Fig. 1A).

We profiled the metabolome of two replicate populations of flies selected for fast (hardy) or slow (susceptible) recovery from chill coma, at three time points: before cold exposure, at the end of 3h cold exposure, and after 5 min recovery (Fig. 1B). We used NMR spectroscopy to quantify the 39 most abundant metabolites of the water-soluble metabolome (all metabolites that could be identified with good confidence and had peaks above the level of baseline noise, Fig. S3, Table S1). Univariate linear models testing the effects of cold exposure, selection and their interaction on each metabolite (accounting for selection population) illustrated a robust metabolic response to both cold exposure and selection (Table 1, Fig. 1C). Selection elicited the largest response, with 23/39 metabolites showing a significant effect of selection and generally large F ratios (Table 1). The largest fold changes were 3-hydroxykynurenine, aspartate, and tyrosine; all higher in susceptible flies. Conversely, dimethylamine, phosphorylcholine, threonine and methionine sulfoxide were higher in hardy flies (Fig. 2A). There were 11 cold-responsive metabolites (significant main effect of cold exposure, Table 1, Fig. 1C), of which three also showed an effect of selection (Table 1). 3-hydroxykynurenine, aspartate, glutamate and malate responded differently to cold stress between hardy and susceptible flies (interaction between cold exposure and selection, Fig. 3, Table 1). Hardy flies regulated levels of 3-hydroxykynurenine, aspartate, glutamate and malate more tightly than susceptible flies during cold exposure (Fig. 3). There was limited overlap between effects of cold exposure and selection – many compounds that responded to selection were not cold responsive and vice versa. Eleven metabolites showed no effect of either cold exposure or selection regime.

To reduce dimensionality of the data and test for differences in metabolite regulation by hardy and susceptible flies during cold exposure, we performed principal component (PC) analysis. PC1 (24.4% of total variance) predominantly captured evolved differences between hardy and susceptible flies (main effect of selection: $F_{1,91}$=22.6, p<0.0001), with hardy flies having lower scores on this PC (Fig. 2B). Scores on PC1 declined over the course of a cold exposure ($F_{2,91}$=4.5, p=0.014) with a trend towards this decline being more pronounced for susceptible flies. Most metabolites loaded positively on this PC (i.e. were lower in tolerant flies) with the exception of dimethylamine, methionine sulfoxide, and phosphorylcholine;

the same metabolites that showed the largest fold-change up-regulation in hardy flies. Thus, experimental cold adaptation has pushed the metabolome in the same direction induced by the plastic response to cold stress; towards a general decrease in levels of most metabolites. Interestingly, PC2 explained almost as much variance as PC1 (20.4%), suggesting that there are multiple important processes occurring during cold exposure and selection. PC2 was dominated by the effects of cold exposure ($F_{2,89}$=11.4, p<0.0001), and an interaction between cold exposure and selection ($F_{2,89}$=5.2, p=0.007) indicated that susceptible flies (but not hardy) sharply increased their scores on this PC during recovery (Fig. 2C). The highest loadings on this PC included alanine and lactate, two byproducts of anaerobic metabolism, suggesting that susceptible flies are in an anaerobic state at 5 minutes after cold exposure whereas the hardy flies that recover more quickly are not.

To measure the consistency of response to selection and assess which compounds were responding most strongly, we used a machine learning approach to separate hardy and susceptible flies based on permutations of their metabolite concentrations (Fig. 4). There were 6 pairs of metabolites that completely separated selection regimes (e.g. Fig. 4A), but triplets of metabolites were required to attain a predicted classification accuracy of 100% based on 10-fold cross-validation. There were 15 triplets of metabolites that attained this level of predicted accuracy (Table S2). The 16 metabolites appearing in those triplets are shown in Fig. 4B; 13 of these were also identified as responding to selection by general linear models (Table 1). Phosphorylcholine and taurine appeared in > 50% of these triplets, indicating that hardy and susceptible flies differed substantially in these two metabolites. Other metabolites appearing in more than two triplets were histidine, 3-hydroxykynurenine, sucrose, threonine and tyrosine. These metabolites thus have the greatest power to discriminate between hardy and susceptible flies. We performed a blind validation study to test the ability of these 15 triplets of metabolites to correctly classify hardy and susceptible flies. We correctly assigned all 12 unknown samples of flies as hardy or susceptible when using a majority vote from all 15 of these triplets (Table S3). The flies used for the validation study were not from an identical population as the training set because they were raised and sampled some months later. This likely explains the inability of some triplets to correctly classify all samples. However, the majority of triplets classified all samples correctly, and the majority of samples were correctly classified by all triplets, demonstrating that evolved differences in the metabolome are robust, repeatable and stable over time.

MAINTENANCE OF METABOLIC HOMEOSTASIS

We used a time series clustering algorithm to identify main trends in metabolite concentrations over the course of cold exposure separately for hardy and susceptible flies. Because we filtered metabolites that did not change above a threshold fold-change or did not show a sufficient correlation between biological replicates of each selection line, metabolites that were placed into a profile were those showing a marked cold response that was consistent across replicates of the selection procedure (originating from two separate base populations). Many more metabolites showed such a response in susceptible compared to hardy flies (19 compared to 9), indicating that metabolite concentrations in hardy flies were perturbed less by cold exposure than metabolites in susceptible flies (χ^2 =4.96, d.f.=1, p=0.026; Fig. 5). There was partial but not complete overlap between metabolites identified

as cold responsive in this analysis and the set of metabolites showing a significant effect of cold in the general linear mixed models (Fig. 5, Table 1). Hardy flies had one significantly enriched temporal profile (Fig. 5A); a set of 4 metabolites that increased during cold exposure and remained elevated during recovery. This profile was not enriched in susceptible flies; only two metabolites showed this temporal response (Fig. 5B). Susceptible flies also had one significantly enriched temporal profile, consisting of metabolites whose levels remained constant or increased slightly during cold exposure, but dropped precipitously during recovery (Fig. 5D). There were no metabolites in hardy flies that fit this profile (Fig 5C). These metabolites are candidates for participating in pathways that are disrupted by cold in susceptible but not hardy flies. Notable within this set were ADP, the product of ATP dephosphorylation; malate, a tricarboxylic acid cycle intermediate; and methionine sulfoxide, the oxidized form of methionine. All other profiles containing more than one metabolite from either hardy or susceptible lines are presented in Fig. 5 E–J – note that all profiles contain more metabolites in susceptible than hardy flies. Thus, the metabolome of susceptible flies is much more disrupted by cold exposure than cold-hardy flies, with hardy flies maintaining metabolic homeostasis more effectively during cold exposure. Aspartate (Fig. 5F) is notable in that it shows a very strong increase during cold in susceptible, but not hardy flies.

STRUCTURE OF METABOLIC NETWORKS

Moving beyond a metabolite-by-metabolite approach, we interrogated the structures of metabolic networks during cold exposure to identify sub-networks that are altered between hardy and susceptible flies, and to form hypotheses about the functional consequences of observed network alterations. Partial correlation networks revealed that metabolic interactions were disrupted by cold exposure with networks becoming smaller, with less nodes and edges, decreased average path-lengths, and weaker partial correlations (Table 2, Fig. 6) in both hardy and susceptible flies. However, the metabolic networks of hardy flies were in general larger and more connected than those of susceptible flies before cold exposure, more correlations were maintained in hardy flies during cold exposure, and hardy flies also recovered the connectivity of their metabolic correlation networks to a far greater extent during 5 min recovery from cold than cold-susceptible flies (Table 2, Fig. 6).

We assessed which correlations among metabolites were perturbed or stable between hardy and susceptible flies. Most of the correlation structure was shared between hardy and susceptible flies (94.4%), but there were 42 correlations that were significantly altered by selection on cold tolerance. The differential correlation networks, composed of the change in Pearson's correlation coefficient for correlations that were significantly altered between hardy and susceptible flies, clearly showed that the correlation structure of a set of key metabolites (histidine, maltose, 3-hydroxykynurenine, and AMP) was highly altered by selection on cold tolerance (Fig. 7A,B). Metabolites whose positive correlations with histidine became stronger in hardy flies are shown in the top right corner of Fig. 7A, including NAD+, phosphorylcholine, β-alanine, methionine sulfoxide, dimethylamine, and lysine (Fig. 7A). Conversely, on the bottom right corner are metabolites whose positive correlations with histidine became stronger in susceptible flies: formate, acetate, leucine, propionate and valine (Fig. 7A). This suggests that pathways or processes linking histidine

with the first set of metabolites are up-regulated in hardy flies, and the second set down-regulated. In the center of that column are 4 metabolites whose direction of correlations with histidine were reversed (Fig. 7A) – isoleucine was significantly and strongly positively correlated with histidine in susceptible flies but uncorrelated in hardy flies, while kynurenate, kynurenine and ADP were significantly and positively correlated with histidine in hardy flies but weakly negatively correlated in susceptible flies. Maltose stands out for having a very high proportion of correlations whose direction was completely reversed by selection on cold tolerance (Fig. 7A), suggesting that regulation of carbohydrate metabolism was remodeled by selection on cold tolerance. Leucine, valine, isoleucine, propionate, acetate, arginine and formate had positive correlations with maltose in hardy flies but negative correlations in susceptible; while 3-hydroxykynurenine, kynurenate, kynurenine and ADP had negative correlations with maltose in hardy flies but positive in susceptible.

Discussion

IMPACTS OF COLD ADAPTATION ON METABOLIC PROFILES

Selection on chill-coma recovery time induced large-scale alterations to metabolic profiles of flies. In general, both cold exposure (plastic changes) and cold adaptation (evolutionary changes) reduced metabolite levels. Previous work has documented an improvement of metabolic homeostasis in *Drosophila melanogaster* in response to cold acclimation (Overgaard et al. 2007; Colinet et al. 2012), and a divergence of the basal metabolome in lines artificially selected for cold-shock resistance (Malmendal et al. 2013). Our work extends beyond these previous studies by demonstrating that there is heritable variation in the ability to maintain homeostasis in the cold that can respond to selection. Better maintenance of metabolic homeostasis was accompanied by a reduced accumulation of anaerobic metabolites during recovery in cold-adapted flies. Basal metabolic networks were larger and more connected in hardy compared to susceptible flies; this may reflect true biological differences in the higher-order network structure related to cold adaptation, or may result from metabolic networks being more divergent in susceptible compared to hardy flies because the two replicate populations of selection lines were pooled (i.e., there may be more ways to be slow at recovering from cold than there are to be fast). Cold-adapted flies had more robust metabolic networks during cold exposure with longer path-lengths and higher connectivity, and cold-induced alterations to specific sub-networks were consistently associated with changes in cold hardiness across lines. In particular, we have identified five metabolic processes that are altered by selection on cold-hardiness that will be targeted for future functional work.

Membrane lipid synthesis and homeoviscous adaptation—The function and integrity of biological membranes depends on their fluidity, and low temperatures can induce a transition from a liquid crystalline to solid gel phase wherein integrity and function are compromised (Jacobson and Papahadjopoulos 1975). Ectotherms modulate the composition of their cellular membranes to maintain fluidity across a range of body temperatures in a process called homeoviscous adaptation (Sinensky 1974). One mechanism of homeoviscous adaptation to low temperatures includes increasing the proportion of phosphatidylethanolamine (PE) head groups (at the expense of more ordered, and thus less

fluid, phosphatidylcholine [PC] head groups) (Hazel 1995). In insects, this process occurs plastically in response to short-term acclimation (Overgaard et al. 2005; Overgaard et al. 2008; Koštál et al. 2011a) and longer-term seasonal acclimatization (Tomčala et al. 2006; Overgaard et al. 2008), although the generality of this mechanism of cold adaptation is not universal (MacMillan et al. 2009). Phosphorylcholine is an intermediate in the pathway between PC and PE, and was among the most altered metabolites in response to selection in our study (Table 1, Fig. 2, Fig. 4B). Cold-hardy flies had higher levels of phosphorylcholine for their body mass than did cold-susceptible flies, in contrast to the pattern for most metabolites that were generally higher in susceptible flies. This observation is consistent with increased flux between PC and PE in cold-hardy flies, which could represent an increased capacity to rapidly modify phospholipid headgroups to maintain functional levels of membrane fluidity in the cold. Phosphorylcholine has been previously demonstrated to increase with cold exposure in *Drosophila melanogaster* (Overgaard et al. 2007), and selection on chill coma recovery time alters membrane phospholipids (Goto et al. 2010). Future experiments will explicitly focus on the lipidome to further assess homeoviscous adaptation hypotheses in these lines and allied naturally derived genotypes that differ in cold-hardiness.

Tryptophan metabolism—The critical aromatic amino acid tryptophan is metabolized primarily through the kynurenine pathway that metabolizes tryptophan into kynurenine, and then into either kynurenate (kynurenic acid) or 3-hydroxykynurenine (Stone and Darlington 2002). These biologically active compounds are integral to diverse processes including neurotransmission, resistance of oxidative stress, cell growth and division, aging, and vision (Stone and Darlington 2002). In particular, 3-hydroxykynurenine is neurotoxic in *Drosophila melanogaster*, while kynurenate is neuroprotective (Campesan et al. 2011). We found that 3-hydroxykynurenine was strongly reduced and less cold responsive in hardy flies (Fig. 3A, Table 1), and was one of the metabolites that most strongly discriminated between hardy and susceptible flies (Fig. 4B). This supports the results of a previous study that demonstrated cold-induced reductions in 3-hydroxykynurenine in *D. melanogaster* (Overgaard et al. 2007). Kynurenate, kynurenine, and 3-hydroxykynurenine were all differentially regulated during cold exposure in hardy compared to susceptible flies, suggesting that pathways involving these metabolites were modulated by cold adaptation (Fig. 7A), and were all in the top third most differentially regulated metabolites between hardy and susceptible flies (Fig. 7B). There were direct correlations involving kynurenate, kynurenine, and 3-hydroxykynurenine at all points in the cold exposure in hardy flies, but there were no correlations between these three metabolites and any other metabolites in susceptible flies (Fig. 6). These complementary lines of evidence support the hypothesis that tryptophan metabolism is altered by selection on cold tolerance. This is in line with previous widespread cold-induced disruptions to tryptophan metabolites (Overgaard et al. 2007). Disruptions to tryptophan metabolism may alter neurotransmission and induce oxidative stress. Pharmacological or genetic manipulations of tryptophan metabolites will be required to directly test these hypotheses, and this represents an exciting new avenue for research.

Energy homeostasis—Maintaining cellular energy levels is a key challenge for ectotherms at temperatures beyond their critical thermal limits for locomotion, and sustained

exposure to temperatures below the critical thermal minimum for locomotion can disrupt energy supply or use. Across multiple taxonomic kingdoms, extreme cold-adaptation improves energy homeostasis at low temperatures, and ATP levels are higher in cold-adapted ectotherms (Napolitano and Shain 2004; Amato and Christner 2009). Consistent with this view, elevated metabolic rates and increased ATP levels are associated with increased cold tolerance in *Drosophila melanogaster* with a mutation in the dystroglycan gene (Takeuchi et al. 2009). We measured ADP and AMP – ATP may have been present but was not possible to quantify due to overlap of critical resonances with some resonances from ADP/AMP. Contrary to the hypothesis that cold adaptation would result in raised adenylate levels, both ADP and AMP were slightly but significantly lower in hardy flies (Fig. 2, Table 1). However, hardy flies maintained their adenylate levels throughout cold exposure, while susceptible flies experienced a drop during recovery (Fig. 5D). The improved adenylate buffering capacity of hardy flies may result from alterations to the phosphoarginine pool; a phosphorylated arginine molecule that serves as a rapid energy reserve in invertebrate muscle tissue (the invertebrate analog of phosphocreatine) (Lewis and Fowler 1962). Future work will examine the dynamics of high-energy adenylate molecules and the phosphagen pool during cold exposure *in vivo*. We also documented a drop in malate and fumarate (tricarboxylic acid [TCA] cycle intermediates) in susceptible but not hardy flies, which could indicate a stalling of the TCA cycle during recovery in susceptible flies. TCA cycle intermediates also decreased during cold exposure in a previous study in *D. melanogaster* (Overgaard et al. 2007). Succinate (another TCA cycle intermediate) increased in hardy flies during cold exposure and recovery, consistent with maintenance of TCA cycle activity in hardy flies. Low temperatures can cause the shutdown of mitochondrial metabolism requiring organisms to rely on anaerobic glycolysis for energy, but it appears that in *D. melanogaster* one of the consequences of cold adaptation is an increased ability to maintain aerobic metabolism in the cold. We are currently testing this hypothesis using stable isotope tracers to measure flux through the TCA cycle and related pathways.

Oxidative stress, protein glycation, and pH buffering—Beyond a long history of study in biomedicine, over the last decade oxidative stress has been increasingly recognized as important in ecological and evolutionary processes from sexual selection to environmental adaptation (Monaghan et al. 2009). We observe several patterns in these data that are consistent with cold-adaptation altering oxidative stress pathways. Histidine can scavenge reactive oxygen species and inhibit oxidative damage (Wade and Tucker 1998; Lemire et al. 2010), and its derivative carnosine (β-alanyl-L-histidine), a dipeptide of histidine and β-alanine, has diverse physiological roles including inhibiting oxidative damage, preventing glycation of proteins, and intracellular pH buffering (Smith 1938; Reddy et al. 2005). In the present study, we found that histidine was consistently and markedly higher in hardy compared to susceptible flies (Fig. 2, Table 1). Histidine has been previously shown to increase in flies selected for resistance to cold shock and other stresses (Malmendal et al. 2013), suggesting that this may be a common component of stress adaptation. β-alanine dropped during recovery from cold in our susceptible flies but not hardy flies (Fig. 2D), and decreases in response to cold exposure in *Sarcophaga crassipalpis* (Michaud and Denlinger 2007). We found that the correlation between histidine and β-alanine was markedly strengthened in hardy flies through cold exposure and recovery (these

metabolites were uncorrelated in susceptible flies, and strongly positively correlated in hardy flies). A strengthened correlation between metabolites could indicate that they are participating in a shared pathway (Steuer 2006). Indeed, histidine was the metabolite whose correlation structure was most altered by selection on cold hardiness with 16 altered correlations, and β-alanine had 3 altered correlations (Fig. 7). It is noteworthy that most correlations between histidine and other metabolites were not only altered in strength, many actually reversed in direction (greyscale cells, Fig. 7A). Multistationarity, or the existence of more than one stable state of regulation, is a common feature of cellular metabolic regulation and indicates that a switch has occurred between metabolic states (Morgenthal et al. 2006). Taken together, these observations strongly implicate histidine and β-alanine as being important to cold adaptation, and suggest that cold adaptation has induced a substantially different metabolic state of regulation in these metabolites. We hypothesize this involvement to be based on their functions as modulators of oxidative stress and protein glycation.

Oxidative stress results from an imbalance between production of reactive oxygen species, and their detoxification by antioxidant molecules (Monaghan et al. 2009). Reactive oxygen species (predominantly superoxide anion, O_2^-; hydroxyl radicals, -OH; and hydrogen peroxide, H_2O_2) are produced by mitochondria as byproducts of oxidative phosphorylation, and unless they are detoxified by antioxidant defenses will inflict oxidative damage on proteins, lipids, and DNA (Monaghan et al. 2009). Detoxification occurs predominantly through a series of reactions that convert superoxide anions to water, generating oxidized glutathione that must then be recycled by reducing equivalents from the reduced coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) generated through the pentose phosphate pathway. The reduction of glutathione seems to be less effective at low temperatures (Joanisse and Storey 1998; Lalouette et al. 2011), leading us to hypothesize that cold-hardy flies may be relying more heavily on alternative pathways (perhaps involving carnosine) for the neutralization of reactive oxygen species. Taurine is also strongly altered as a consequence of cold adaptation (Fig. 4B). Taurine is a cysteine derivative, and has diverse physiological roles that include neuroprotection and neuromodulation (Olive 2002), antiglycation (Huang et al. 2008), and antioxidant function (Zhang et al. 2004).

Carnosine and taurine may also prevent protein glycation, which may be of increased concern at low temperatures (Storey 2004). Glycation occurs when reducing sugar molecules haphazardly form covalent bonds with proteins or lipids, forming advanced glycation end products (AGEs) which have pro-oxidant actions and have been implicated in diverse pathologies (Reddy et al. 2005). Although sugars are accumulated to high concentrations in overwintering or cold-acclimated insects (Overgaard et al. 2007; Koštál et al. 2011a; Colinet et al. 2012), when reducing sugars are supplemented to the diet they negatively impact cold tolerance in *Drosophila melanogaster* (Colinet et al. 2013). Carnosine and taurine, in their roles as glycation inhibitors (Reddy et al. 2005; Huang et al. 2008), may increase cold tolerance by decreasing the propensity for proteins to aggregate at low temperatures. Further research is needed to clarify the thermal dependence of AGE formation and its role in chilling injury in ectotherms.

Proline metabolism—Proline is an important cryoprotectant in insects (Lee 2010), and is one of few molecules for which a causative relationship with cold-tolerance in insects has been demonstrated (Koštál et al. 2011c). We thus predicted that proline would be higher in cold-hardy flies. Contrary to our prediction, proline was significantly lower in hardy flies (Table 1), but the magnitude of effect size was very small and proline was not a good predictor of cold tolerance (Fig. 4B). Proline was cold-responsive, dropping upon exposure to cold in both hardy and susceptible flies, but its behavior during recovery differed among selection regimes; proline homeostasis was restored within 5 min recovery in hardy flies (Fig. 2G), while levels continued to drop precipitously in susceptible flies (Fig. 2H). This suggests proline homeostasis, rather than basal levels, was altered by selection on cold tolerance, with tighter regulation in hardy flies. Proline is used as a fuel for flight and other energetically expensive metabolic processes in insects (Scaraffia and Wells 2003), thus improved proline homeostasis in hardy flies may indicate generally improved energetic homeostasis.

EXTRAPOLATING MECHANISMS FROM EXPERIMENTAL EVOLUTION EXPERIMENTS

Experimental evolution by laboratory artificial selection has repeatedly shown utility for identifying components of the genetic and physiological/mechanistic architectures of phenotypic evolution (Gibbs 1999; Burke and Rose 2009; Zera 2011). However, the evolutionary trajectories followed by artificially selected lines may not reflect responses of naturally occurring populations during local adaptation due to numerous factors (Gibbs 1999). A key factor when considering experimental evolution from the perspective of the metabolome is that there physiological costs of altered metabolism may be low in a benign laboratory environment (Huey and Rosenzweig 2009). Changes to intermediary metabolism that increase ATP demand and require a higher rate of food consumption may not be selected against in an environment with constant food available *ad libitum*, but may have costs that prevent their evolution in natural populations. Thus, additional work is needed to determine whether our observation of greater metabolic homeostasis in cold-hardy genotypes holds in isolates from natural populations.

This study is also subject to limitations that apply to all “omics” studies of metabolism (Suarez and Moyes 2012); measurements are taken at a snapshot in time, and as such cannot truly provide inferences about dynamic metabolic processes that result from nutrient fluxes. We have leveraged correlations among metabolites that interact via the metabolic network to infer alterations to metabolic pathways, but no true inferences on metabolic flux are possible without using combinations of techniques such as stable isotope tracer studies and respirometry (Wolfe and Chinkes 2004; Lighton 2008). Although most ‘omics studies will generate far more hypotheses than will ever be explored, they remain a powerful tool.

CONCLUSIONS

Here we show that cold adaptation alters metabolic profiles of flies prior to cold exposure, during cold stress, and during recovery from cold stress. Cold-hardy lines had greater resistance to perturbation of both metabolite levels and the structure of metabolic networks during cold exposure, and greater tolerance to cold-induced perturbations of metabolism as reflected in faster recovery of levels of perturbed metabolites and rapid recovery of

metabolite networks. We show evidence suggesting alterations to phospholipid, tryptophan, and proline metabolism; energy balance; and oxidative stress and protein glycation. These are promising avenues for targeted experiments to examine the impacts of cold-adaptation on metabolic flux through these pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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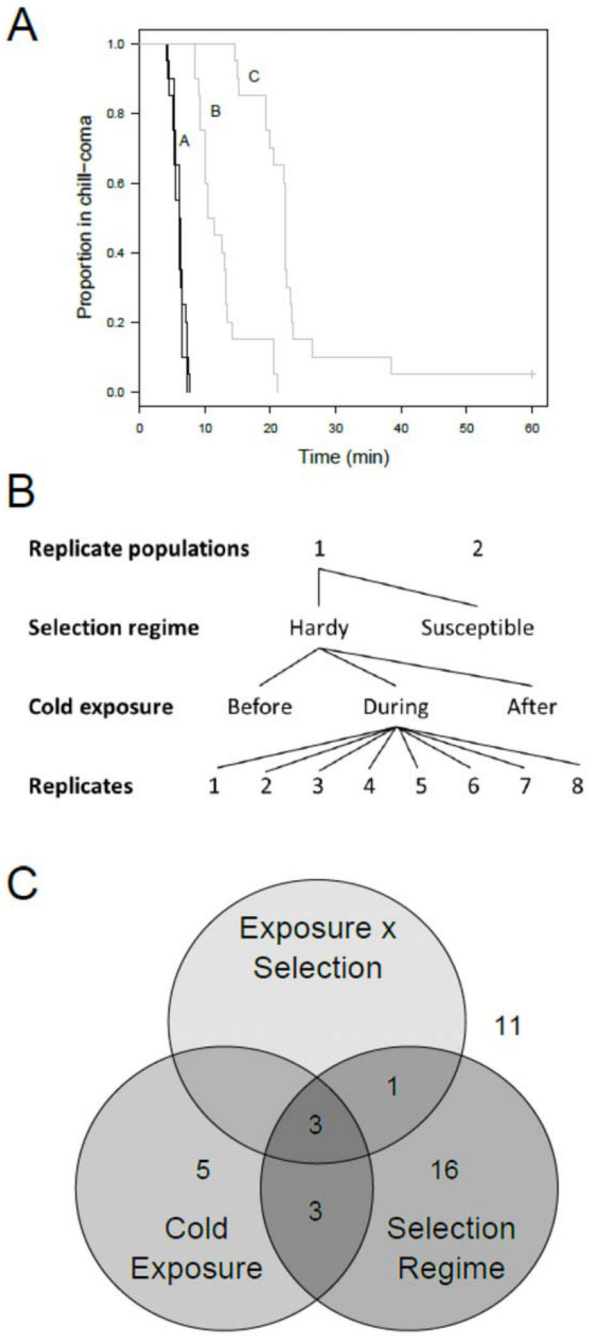

Figure 1.

Summary of the response to cold exposure and selection for cold tolerance. A) Proportion of 20 flies still in chill coma as a function of time in replicate lines of cold-hardy (black) and cold-susceptible (grey) *Drosophila melanogaster* at the time of collection of the samples for the metabolomics experiment. Letters indicate lines that are significantly different according to an accelerated failure time model with a log-logistic error distribution, and the + indicates one censored value – a single fly from one of the susceptible lines did not wake up within 60 min. Hardy lines are statistically indistinguishable from each other, and have faster recovery

than either of the susceptible lines. B) Experimental design of the metabolomics experiments. C) Numbers within each circle indicate the corresponding number of metabolites displaying a significant main effect of cold exposure, selection regime, or an interaction between the two. The number outside all circles indicates metabolites that did not respond to selection or cold exposure.

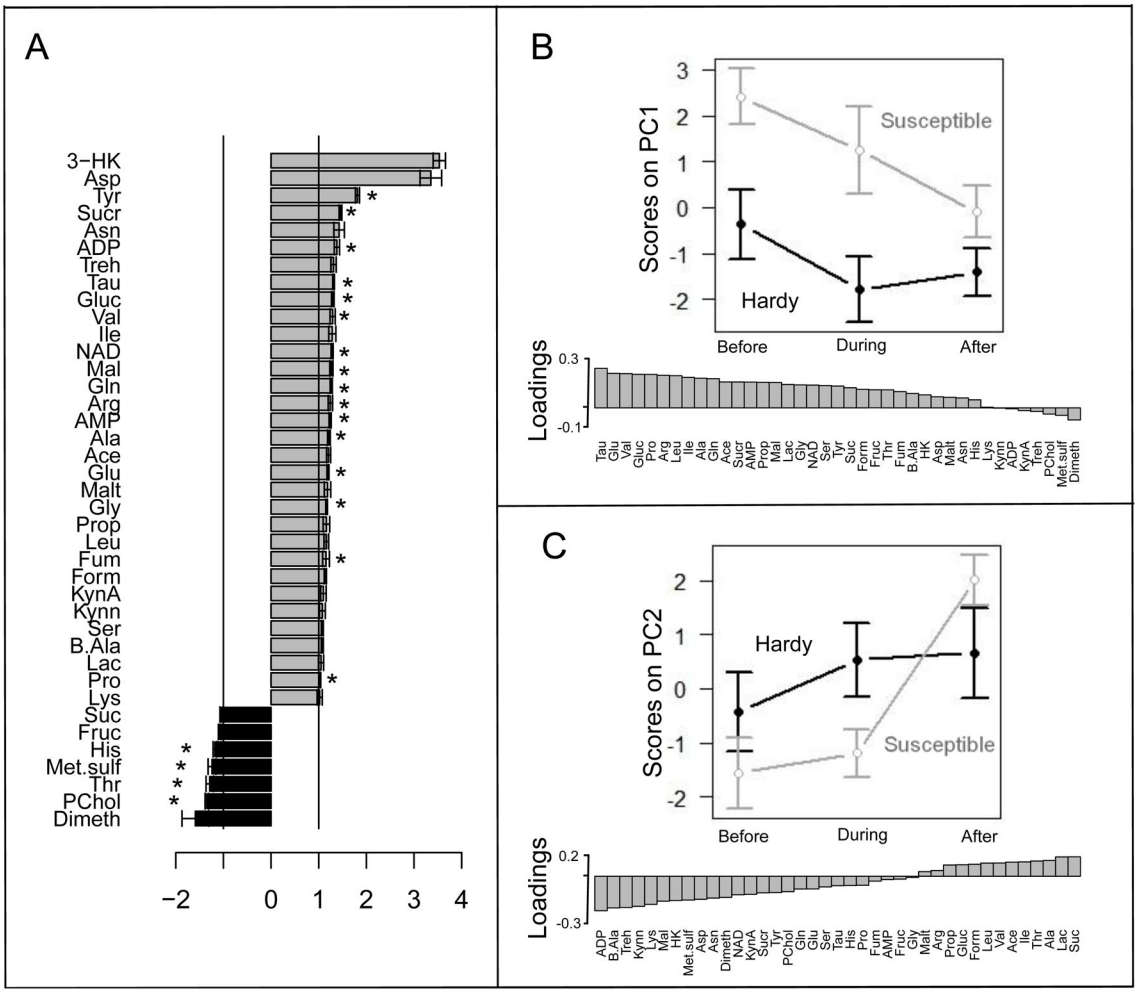

Figure 2.

Changes in metabolite content of *Drosophila melanogaster* resulting from selection for fast or slow recovery from chill coma (hardy and susceptible respectively). A) Fold changes of metabolites from hardy (black) or susceptible (grey) flies, pooled across all time points. Asterisks indicate significant differences between groups (false discovery rate < 0.05; Table 1). B–C) Principal component scores (B= PC1, C=PC2), and associated loadings of each compound onto those components, for flies sampled before cold exposure, at the end of a 3h cold exposure (during), or after 5 min recovery (after).

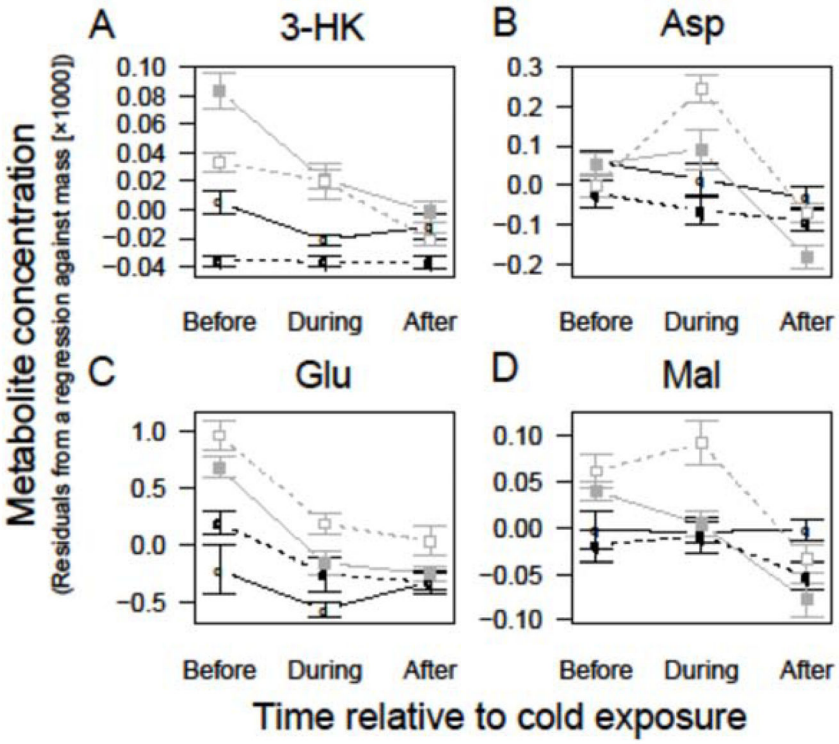

Figure 3.

Select metabolite concentrations before, at the end of a 3h cold exposure, or after 5 minutes recovery from a 3h cold exposure for cold-susceptible (grey) or –hardy (black) *Drosophila melanogaster* for metabolites that show an altered cold responsiveness in cold hardy compared to susceptible flies (interaction between cold exposure and selection, Table 1, Fig. 1B). Line style delineates biological replicates of the selection lines – solid is replicate 1, dotted replicate 2. Hardy flies have lower metabolite concentrations, and maintain better homeostasis during cold exposure. Values are mean ± SEM.

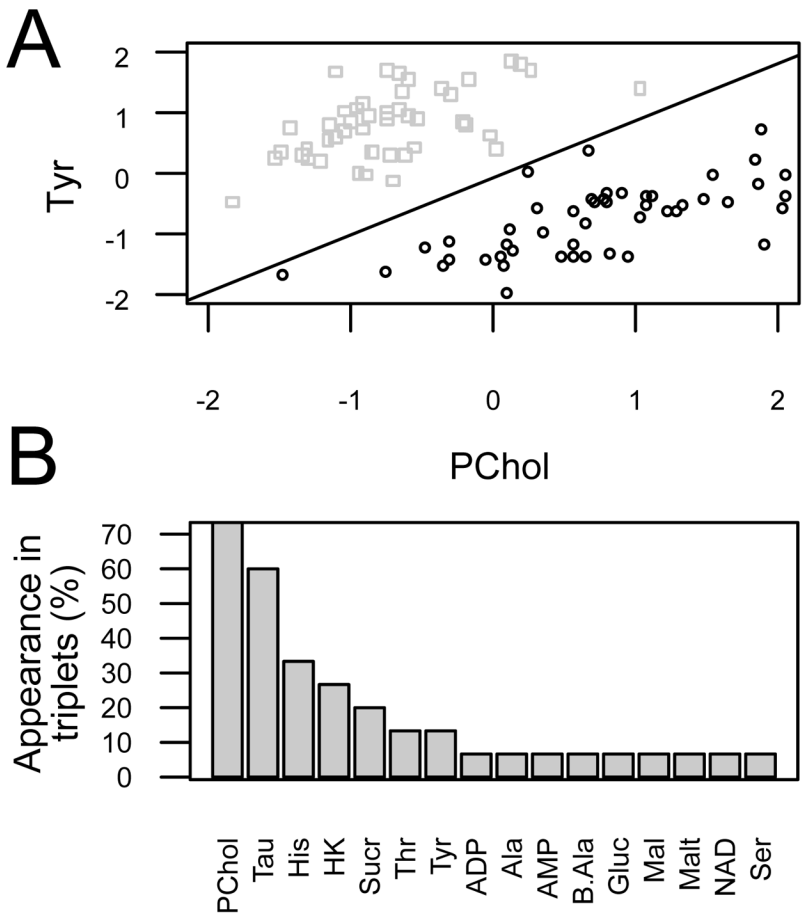

Figure 4.

Classification of cold hardy and susceptible lines using support vector machines. A) An example of a two compound separation, with the equation of the separating vector (the support vector) given at the top of the figure. B) The percent appearance of each metabolite in the 15 triplets of metabolites that were predicted to give 100% accuracy in classifying unknown flies as hardy or susceptible based on 1000 replicates of 10-fold cross-validation. Metabolites that did not occur in any of those 15 triplets are not shown.

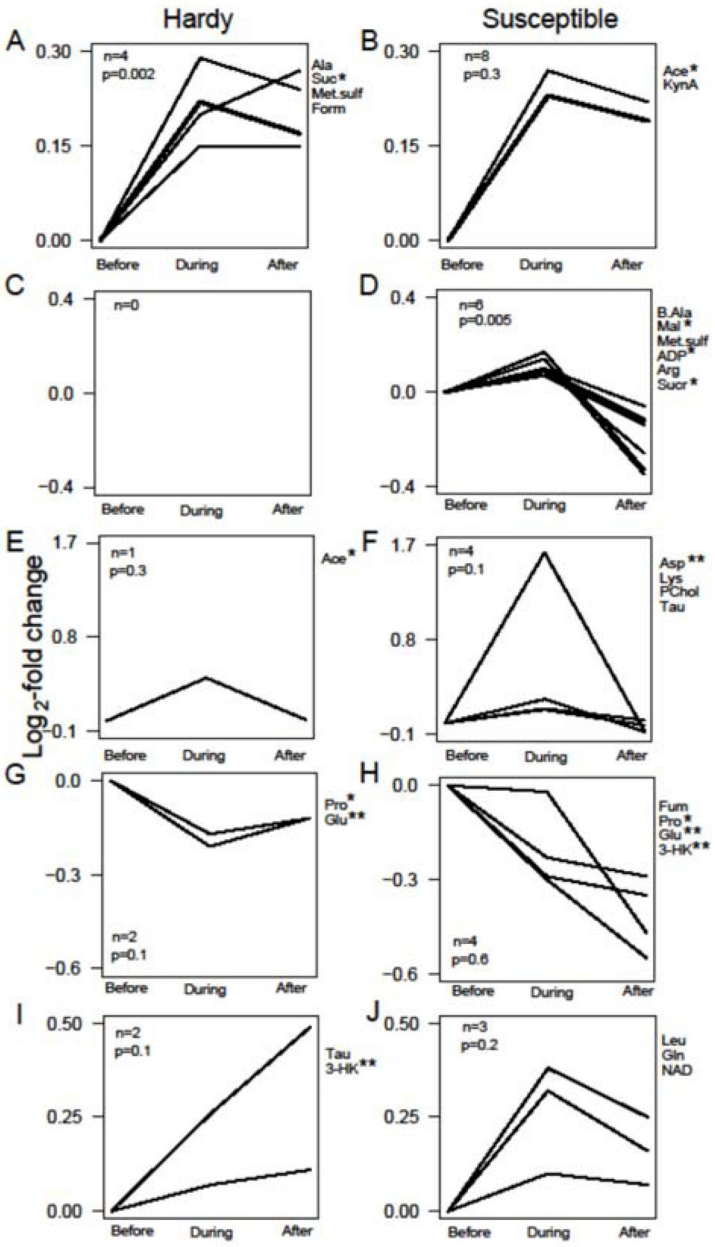

Figure 5.

Cold response of metabolites from cold hardy or susceptible *Drosophila melanogaster*. Metabolites were sampled before cold exposure (before), at the end of a 3h cold exposure (during), or after 5 minutes recovery (after), and grouped according to their temporal expression pattern. Hardy flies had less cold-responsive metabolites (9 compared to 19), and thus maintained metabolic homeostasis more effectively during a cold exposure. Asterisks indicate metabolites that also have a significant main effect of cold exposure from linear models; double asterisks those with altered cold responsiveness between selection lines (Table 1).

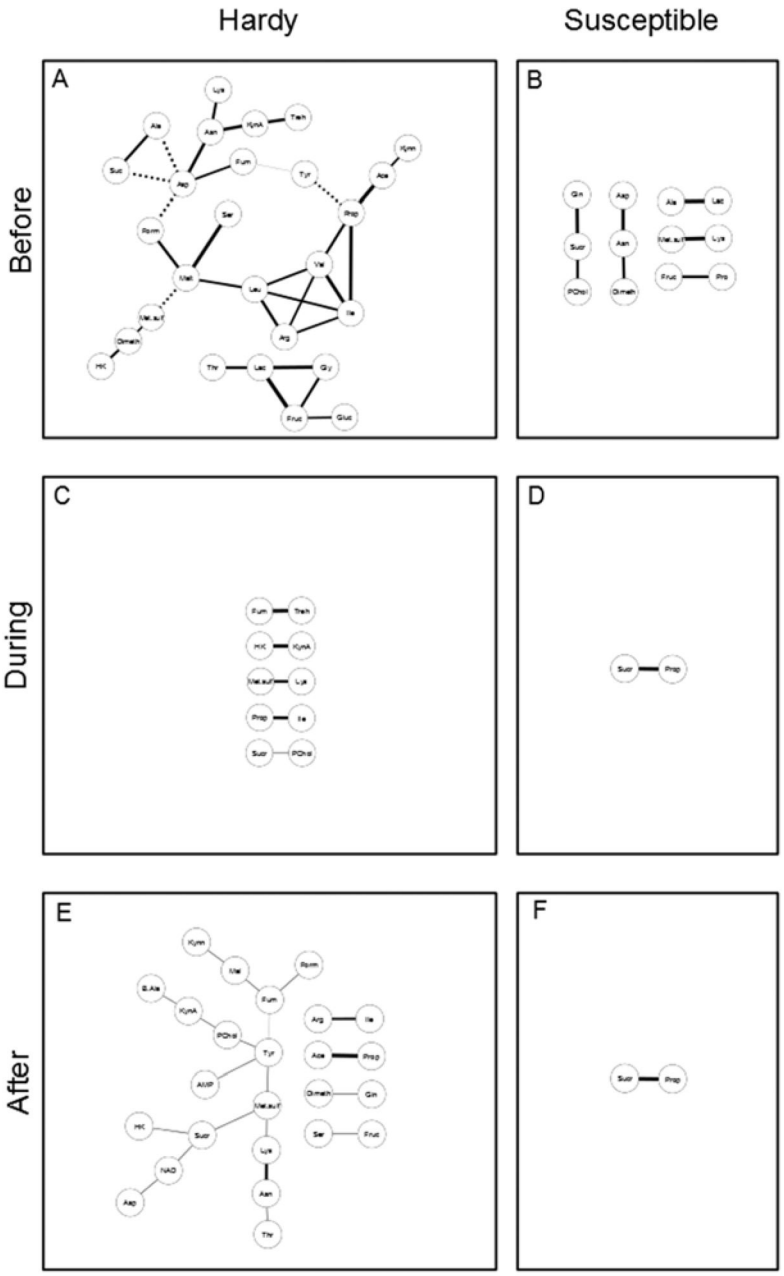

Figure 6.

Partial correlation networks for cold-hardy (A,C,E) and –susceptible (B,D,F) flies before (A,B), during a 3h cold exposure (C,D) and after 5 minutes recovery (E,F). Nodes represent metabolites; edges indicate a significant first-order partial correlation ($Q < 0.1$) between those two metabolites, weighted for the strength of the correlation. Negative partial correlations are shown by a dotted edge. Hardy flies have more correlations among metabolites than do susceptible flies, and these correlations are restored after cold while for susceptible flies the correlation structure is lost.

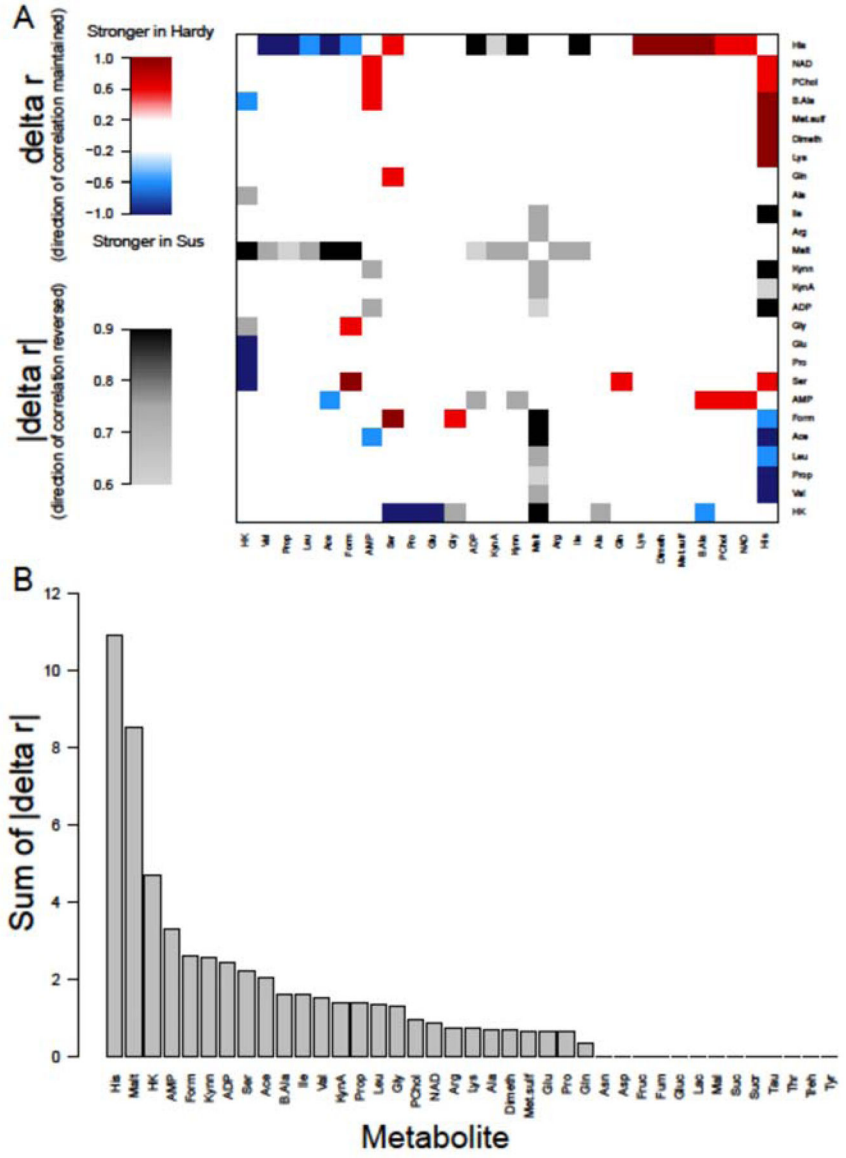

Figure 7.

Correlations between metabolites that are altered by selection for cold tolerance. Metabolites whose correlation structure was completely conserved were removed from the matrix. Correlations that are conserved between tolerant and susceptible flies are white; altered correlations that retain the same sign (positive or negative correlation) are in color, while correlations whose sign is reversed are in greyscale. B) Sum of the absolute value of delta r for each metabolite against every other metabolite. Large values indicate metabolites whose correlation structure is highly altered by selection on cold tolerance.

Table 1

The effect of cold exposure and selection for cold-tolerance of levels of metabolites in *Drosophila melanogaster*, measured using ^{1}H NMR spectroscopy. Flies were selected for fast or slow recovery from chill coma (selection regime), and sampled either before, during or after a 3h cold exposure (cold exposure). To test the effect of our experimental design on individual metabolites, we used general linear models with the fixed factors of cold exposure, selection regime, and their interaction, with biological replicate of selection line modeled as a random factor, and corrected the p-values using a false discovery rate correction (Benjamini and Hochberg 1995). Effects that are significant at $Q < 0.05$ are in boldface. Degrees of freedom are 1 (main effects) or 2 (interaction) with 88 residual degrees of freedom. Metabolites are ordered by the sums of F statistics across rows, thus are ranked according to degree of perturbation by the combined experimental treatments. Selection regime caused the greatest alterations to metabolite levels.

		Cold Exposure		Selection regime		Exposure × Selection	
Metabolite	**Abbr.**	**F**	**Q**	**F**	**Q**	**F**	**Q**
3-Hydroxykynurenine	3-HK	4.03	0.061	**177.07**	**<0.001**	**13.37**	**<0.001**
O-Phosphocholine	PChol	2.27	0.185	**130.03**	**<0.001**	1.4	0.421
L-Glutamate	Glu	**27.62**	**<0.001**	**84.54**	**<0.001**	**9.82**	**0.002**
L-Tyrosine	Tyr	1.85	0.256	**111.71**	**<0.001**	0.18	0.838
Taurine	Tau	0.99	0.49	**55.38**	**<0.001**	2.11	0.292
Sucrose	Sucr	**5.83**	**0.015**	**34.74**	**<0.001**	1.33	0.421
L-Aspartate	Asp	**19.92**	**<0.001**	**6.82**	**0.02**	**13.17**	**<0.001**
Nicotinamide adenine dinucleotide	NAD	1.3	0.372	**38.12**	**<0.001**	0.39	0.735
Adenosine diphosphate	ADP	**14.04**	**<0.001**	**18.9**	**<0.001**	3.34	0.194
Malate	Mal	**13.09**	**<0.001**	**11.22**	**0.003**	**7.64**	**0.008**
L-Histidine	His	2.64	0.167	**23.08**	**<0.001**	0.2	0.838
D-Glucose	Gluc	2.43	0.175	**18.68**	**<0.001**	2.27	0.292
L-Alanine	Ala	3.83	0.066	**17.43**	**<0.001**	0.76	0.614
L-Proline	Pro	**8.95**	**0.002**	**7.04**	**0.018**	2.16	0.292
Dimethylamine	Dimeth	3.99	0.061	**11.94**	**0.003**	0.84	0.587
Methionine sulfoxide	Met.sulf	1.47	0.353	**12.45**	**0.002**	2.4	0.29
Acetate	Ace	**8.54**	**0.003**	3.53	0.095	4.01	0.12
Threonine	Thr	3.52	0.082	**10.75**	**0.003**	1.39	0.421
Glycine	Gly	2.43	0.175	**10.79**	**0.003**	0.54	0.692
L-Valine	Val	0.47	0.721	**11.7**	**0.003**	0.65	0.654

		Cold Exposure		Selection regime		Exposure × Selection	
Metabolite	**Abbr.**	**F**	**Q**	**F**	**Q**	**F**	**Q**
Lactate	Lac	**6.76**	**0.009**	4.68	0.054	1.04	0.517
Trehalose	Treh	**6.86**	**0.009**	1.3	0.324	4.05	0.12
L-Arginine	Arg	2.5	0.175	**7.69**	**0.014**	1.86	0.336
Adenosine monophosphate	AMP	0.49	0.721	**10.73**	**0.003**	0.44	0.717
L-Glutamine	Gln	1.33	0.372	**5.39**	**0.038**	2.59	0.261
Succinate	Suc	**6.09**	**0.013**	0.07	0.82	2.93	0.255
β-Alanine	B.Ala	4.02	0.061	0.32	0.603	4.55	0.102
Kynurenine	Kynn	**6.39**	**0.011**	0.01	0.914	1.85	0.336
Fumarate	Fum	0.88	0.527	**5.97**	**0.029**	1.14	0.489
L-Lysine	Lys	2.35	0.18	2.04	0.211	2.12	0.292
L-Isoleucine	Ile	0.18	0.862	4.42	0.06	0.49	0.702
Propionate	Prop	3.36	0.09	0.59	0.511	0.63	0.654
Maltose	Malt	0.34	0.792	2.78	0.143	1.33	0.421
L-Serine	Ser	0.27	0.805	1.41	0.309	2.65	0.261
L-Asparagine	Asn	2.16	0.197	0.78	0.448	1.34	0.421
L-Leucine	Leu	0.31	0.792	0.94	0.407	2.79	0.261
Fructose	Fruc	0.03	0.974	2.2	0.198	0.85	0.587
Kynurenate	KynA	0.51	0.721	0.32	0.603	1.58	0.413
Formate	Form	1.36	0.372	0.35	0.603	0.3	0.783

Table 2

Metabolic network parameters for hardy (shaded) and susceptible (Sus, unshaded) *Drosophila melanogaster* before cold exposure (Before), at the end of a 3h cold exposure (During), and after 5 min recovery (After). Networks are illustrated in Fig. 6.

	Before		During		After	
Network parameters	**Hardy**	**Sus**	**Hardy**	**Sus**	**Hardy**	**Sus**
Number of nodes	27	12	10	2	25	2
Number of edges	35	7	5	1	20	1
Clustering coefficient	0.3	0	0	0	0	0
Network diameter	8	2	1	1	7	1
Characteristic path length	3.7	1.2	1.0	1.0	3.5	1.0
Avg. number of neighbors	2.4	1.2	1.0	1.0	1.6	1.0